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BINDING STUDIES OF EPIDERMAL GROWTH FACTOR RECEPTOR TARGETED COMPOUNDS USING SURFACE PLASMON RESONANCE

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BINDING STUDIES OF EPIDERMAL GROWTH FACTOR RECEPTOR TARGETED COMPOUNDS USING SURFACE PLASMON RESONANCE

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters at Virginia Commonwealth University.

By

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ABBREVIATIONS

CHCA	α-Cyano-4-hydroxy-cinnamic acid
DCM	Dichloromethane
DFM	N, N-Dimethylformamide
DIEA	N, N-Diisopropylethylamine
DODT	3, 6-Dioxa-1, 8-octane-dithiol
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide Hydrochloride
EDTA	Ethylenediaminotetraacetic acid
HBS-EP	HEPES-buffered saline containing EDTA and surfactant P-20.
HBTU	O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic
HOBt	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
Fab	Fragment, antigen-binding
IFC	Integrated fluidic cartridge
IgG1	Immunoglobulin G1

k_a	Association rate constant ($M^{-1}s^{-1}$)
k_d	Dissociation rate constant (s^{-1})
K_D	Equilibrium dissociation constant (M)
MAb	Monoclonal Antibody
MALDI-TOF	Matrix-assisted laser desorption ionization-time-of -flight
MeCN	Acetonitrile
MW_A	Molecular weight of analyte
MW_L	Molecular weight of ligand
NHS	N-hydroxysuccinimide
NMP	1-Methyl-2-Pyrrolidone
R_{eq}	Response at equilibrium
R_L	Ligand immobilization level in RU
R_{max}	Maximum analyte binding capacity in RU
RU	Response Units
sEGFR	soluble EGFR
SPR	Surface Plasmon Resonance
Sm	Stoichiometry
TFA	Trifluoroacetic acid
TGF	Transforming Growth Factor
TIS	Triisopropylsilane
UV-Vis	Ultraviolet – visible spectroscopy

Abstract

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By Spandana Kankanala, B Tech

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Michael H. Peters

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The study of binding kinetics of proteins plays an important role in understanding molecular mechanisms that drive biological processes. The binding rate constants reflect the dynamics of the system and associated biological activity measurements of the association and dissociation rate constants make it possible to compare different interactions in a standardized manner and help elucidate a mechanistic understanding of binding events.

In our study, we used Surface Plasmon Resonance (SPR) technology (Biacore) to study the binding kinetics of the antibodies EGF, Cetuximab and a candidate drug P-13 with the receptor EGFR. The candidate drug P-13 was synthesized and tested on Biacore for binding kinetics. This peptide is anticipated to bind to domain III of EGFR-ED. The study also compared the interaction kinetics of EGF/EGFR and Cetuximab/EGFR with the previous literature and a summary of results is produced.

Our Biacore experiments on EGF/sEGFR suggest a two-state affinity binding with 90% high affinity binding sites, which compares with the previous studies in cells. The dissociation rate constant for Cetuximab/sEGFR interaction was reported for the first time using SPR while the other kinetic constants were comparable to literature. Although the peptide P-13 demonstrated a relatively weak (micro molar) binding capacity to the receptor, as compared with EGF and Cetuximab, the dissociation rate constant was comparable to a nano molar binder. Hence, we argue that the region of binding of P-13 is sterically inhibited as per the receptor orientation, which is consistent with the computer design data supplied with this candidate drug.

CHAPTER 1 Introduction

1.1 Overview

Cancer is one of the major causes of death in the U.S. It starts as the cell begins to divide uncontrollably. This cell multiplication eventually leads to a visible mass called a tumor. Tumor start as benign, which are not fatal, but sometimes change into malignant tumors as characterized by spreading (metastasis) to multiple regions of the body. The type of the cancer is identified by the initial tumor location. There are more than 100 types of cancer, including breast cancer, skin cancer, lung cancer, colon cancer, prostate cancer, and lymphoma (Jemal *et al*., 2008).

Colorectal cancer is the third most common cancer found in U.S. The American Cancer Society estimates that there will be about 108,700 new cases of colon cancer and 40,740 new cases of rectal cancer in 2008. Combined, this might cause 49,960 deaths in U.S alone. Colorectal cancer, cancer that starts either in the colon or the rectum, is treated by surgery, radiation therapy, chemotherapy or targeted therapies called monoclonal antibodies.

Although surgery, radiation therapy, and chemotherapy are suggested treatments for cancer, there are common side effects including bleeding from surgery, damage to nearby

organs during operation, mild skin irritation due to radiations, nausea, diarrhea, rectal and bladder irritation, tiredness. While chemotherapy kills cancer cells, it also damages some normal cells which cause side effects like increased chance of infection, hair loss, easy bleeding or bruising after minor cuts or injuries.

Targeted therapies (monoclonal antibodies) are used to destroy some types of cancer cells while causing little harm to normal cells. They are designed to recognize certain proteins (receptors) that are found on the surface of particular cancer cells. The major criteria for the proteins to be targets for treatment are that they should be accessible to the antibodies and be available during the whole treatment time. In this thesis, we concentrate on EGFR (Epidermal Growth Factor Receptor), the target receptor, whose constitutive activity leads to cell proliferation and, in turn, cancer. EGFR is highly upregulated in colorectal cancers and, thus, has received significant attention as a target for drug development.

1.2 Objective

In this thesis, the binding kinetics of the drug Cetuximab (monoclonal antibody) and the growth factor EGF (Epidermal Growth Factor) with sEGFR (soluble EGFR) were studied using SPR (Surface Plasmon Resonance). The kinetic rate constants and the methods used to obtain values for EGF and Cetuximab (C225) interactions with sEGFR are compared to that in the literature. Our goal is to carry on SPR studies in an

arrangement which would imitate the natural system as closely as possible in order to obtain kinetic constants consistent with cellular-based results.

A thirteen residue peptide, code named P-13, has been proposed as an EGFR inhibitor that binds to residues near 500 of domain III (Peters, 2009). One of the goals of this research is to test P-13 in Biacore and compare its binding to that of EGF and Cetuximab.

CHAPTER 2 Theory

2.1 EGF Pathway: Cell growth and proliferation

The epidermal growth factor receptor (EGFR) family, also called ErbB family, consists of four kinds of receptors, EGFR (ErbB1), Neu (ErbB2), ErbB3, and ErbB4. Each receptor consists of a cytoplasmic tyrosine kinase domain, a single transmembrane spanning region, and an extracellular region, the latter of which contains approximately 620 amino acids. The extracellular region of each receptor has four domains, L1 and L2 (or I and III), which are luecine-rich regions and CR1 and CR2 (or II and IV), which are cystine-rich (Ferguson *et al*., 2003). The ligand EGF binds to the extracellular domain of the receptor and aids in the dimerization of the receptors to activate the signal cascade for gene transcription and cell proliferation.

The EGF receptor has many growth factors, such as Epidermal Growth Factor (EGF) and Transforming Growth Factor-α (TGF-α). EGF when bound to the extracellular domain of EGFR leads to receptor dimerization and activation of tyrosine kinase enzyme located in the intracellular domain of the receptor. Kinase activation leads to transphosphorylation of the receptor intracellular domains and initiates multiple signal transduction pathways that eventually result in the signal reaching the nucleus.

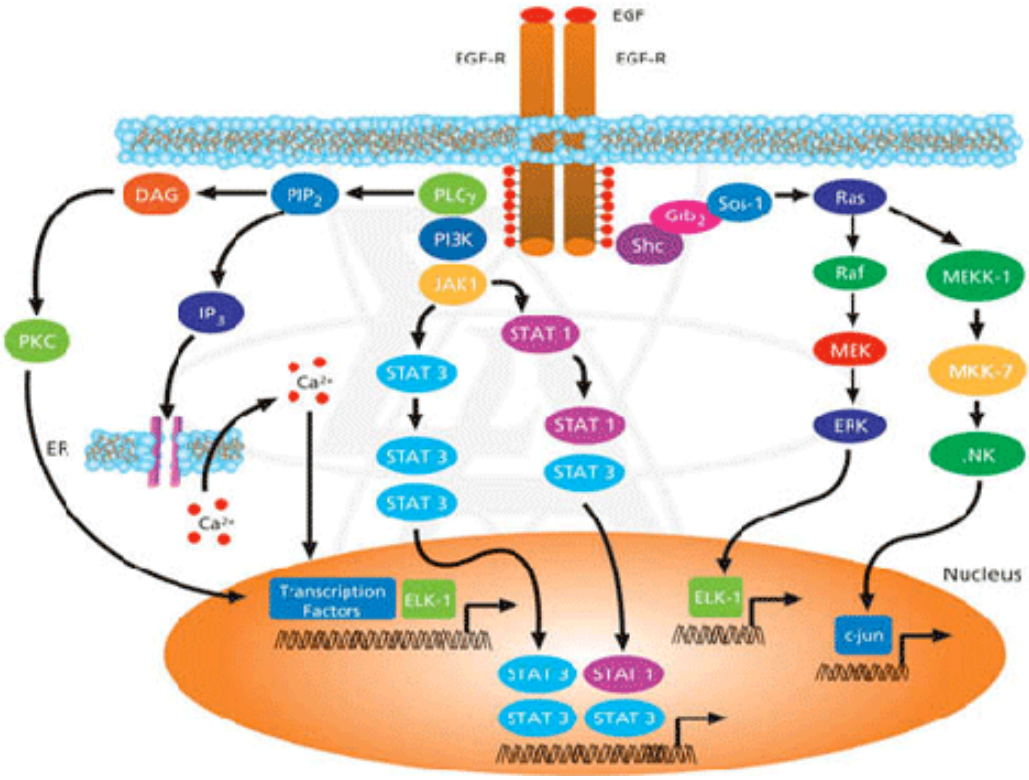

Figure 2.1-1 EGF receptor signal transduction pathway

The Ras-Raf-MEK-MAPK pathway is activated through the growth factor receptor bound protein 2 (GRB2)-SOS complex. The receptor mediated signaling also activates phosphotidylinositol 3-kinase (PI3K)-AKT pathway, which contributes to anti-apoptic effects of EGFR activation. Additionally, transcription activator (Stat) proteins are also activated. These EGFR downstream signaling pathways lead in cellular responses like cell proliferation, differentiation, cell motility, adhesion and angiogenesis. The over expression, mutation or truncation of the EGF receptor leads to constant activity of the receptor and hence leads to excess cell growth and, in turn, cancer. EGFR is implicated in

the development of wide range of epithelial cancers, including those of breast, colon, head and neck, kidney, lung, pancreas and prostate (Scaltriti *et al*., 2006).

2.2 Cancer Research: Antibodies

Recent research has explored promising methods to suppress tumor growth in tissues. Over the last decade, targeted therapies have been proven to be an effective way to inhibit tumors with fewer severe adverse events in cancer intervention since they specifically interfere with signaling pathways essential for tumor growth. Monoclonal antibodies and protein tyrosine kinase inhibitors are two classes among these new therapeutic interventions.

The monoclonal antibodies target the extracellular ligand binding domain of the receptor and prevent the activation of the receptor by its respective growth factor. On the other hand, tyrosine kinase inhibitors are small molecules which mainly target the ATP binding domains of the receptor and thereby prevent phosphorylation of the kinase domain and its activation.

One simple and effective way to treat tumors is the use of monoclonal antibodies along with other cancer therapies or individually. Monoclonal antibodies are produced by forming a hybrid cell that has the ability to produce the desired antibody continuously. In this technology, the tumor cells that can replicate endlessly are fused with mammalian cells that produce the antibody. This results in “hybridoma”, the fused cell, which

continually produces the antibody. The antibodies thus produced are pure as they come from only one type of cell (Schwaber, J *et al*., 1973).

Monoclonal antibodies function in cancer treatment through various mechanisms. They can directly affect the tumor by causing apoptosis or programmed cell death. They can compete with growth factors in binding to receptor domains and thereby arrest signaling for cell proliferation. These antibodies can be also used in the clinic as conjugated antibodies, with a radionuclide or chemotherapy agent attached or as non-conjugated antibodies, without any toxins or radionuclide attached to them.

The FDA (Food and Drug Administration) approved the first monoclonal antibody for cancer treatment in 1997. Today several of these drugs are in market for treating certain cancers. Below is the table of the MAbs approved by the FDA for cancer treatment.

Table 2.2-1 Monoclonal antibodies used to treat cancer (Rang, H.P. *et al*., 2003)

MAb Name	**Trade Name**	**Used to Treat**	**Approved in:**
Rituximab	Rituxan	Non-Hodgkin lymphoma	1997
Trastuzumab	Herceptin	Breast Cancer	1998
Gemtuzumab ozogamicin	Mylotarg	Acute myelogenous leukemia (AML)	2000
Alemtuzumab	Campath	Chronic lymphocytic leukemia (CLL)	2001
Ibritumomab tiuxetan	Zevalin	Non-Hodgkin lymphoma	2002
Tositumomab	Bexxar	Non-Hodgkin lymphoma	2003
Cetuximab	Erbitux	Colorectal cancer Head & neck cancers	2004 2006
Bevacizumab	Avastin	Colorectal cancer Non-small cell lung cancer Advanced breast cancer	2004 2006 2008
Panitumumab	Vectibix	Colorectal cancer	2006

Cetuximab (Erbitux) is an antibody against EGFR protein as it blocks the activation of this receptor. It was approved by FDA in 2004 for treatment of colorectal cancer. Recently, it was approved for the treatment of head and neck cancers (2006).

2.3 Binding studies: EGF/Cetuximab/P-13 with EGF Receptor

Structural studies on EGF-EGFR complex have shown that the growth factor binds to the domains I and III of extracellular-EGFR simultaneously. The unliganded EGF receptor is in its unextended form (inactive state) with domain II buried by an intramolecular interaction with domain IV. When the growth factor binds to the receptor (domains I and III), it alters the spatial arrangement of the domains and this domain rearrangement exposes a critical region, known as the dimerization arm, of domain II. This aids in the dimerization of the receptor and thus activation of cell signaling (Li *et al*., 2005).

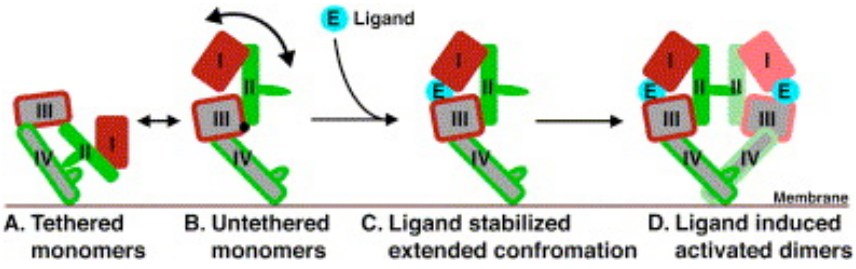

Figure 2.3-1 Model for EGF induced activation of EGFR

Cetuximab, as shown in structural studies, binds exclusively to domain III of the EGF receptor that overlaps the EGF binding site. Thus, it prevents the EGF from binding to the receptor and thereby prevent tumor causing cell signaling (Li *et al*., 2005).

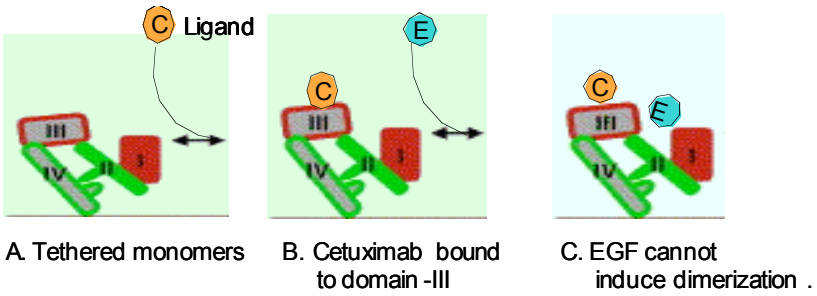

Figure 2.3-2 Model for Cetuximab preventing EGF binding to EGFR

P-13 is a thirteen residue peptide which is anticipated to bind domain III of EGFR extracellular region. The peptide binds to residues near 500 of domain III (Peters, 2009) and Figure 2.3-3 shows the binding regions of peptide on sEGFR.

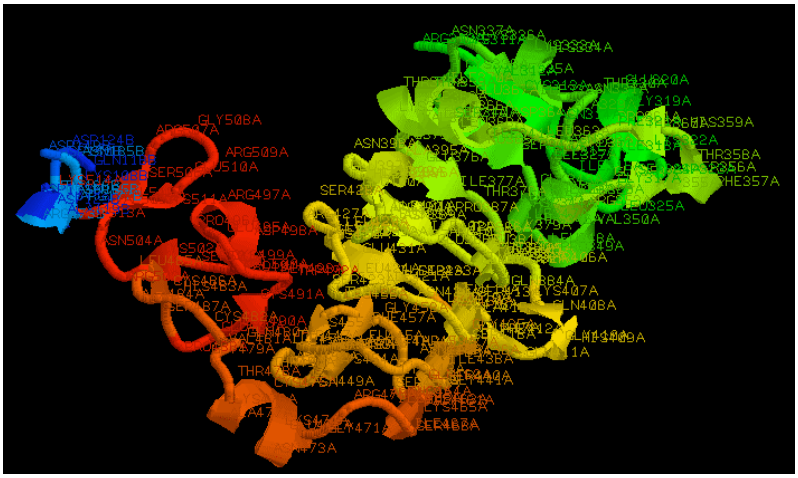

Figure 2.3-3 Structure of P-13 binding to EGFR-ED

CHAPTER 3 Literature Review

3.1 Overview of the Chapter

In this chapter, we review previously published studies on the binding of antibodies EGF and Cetuximab to EGFR. We compare studies carried over in cells with that on Biacore. In the later part of this chapter, important Biacore phenomena are discussed.

3.2 Review of Previous Studies on Binding of EGF to Heterogeneous EGFR

Epidermal growth factor (EGF) is known to bind to its receptor (EGFR) with an equilibrium dissociation constant (K_D) of 1-10nM in living cells. Quantitative binding experiments demonstrated that EGF binds to EGFR with two distinct dissociation constants: a minority (2%–5%) of high-affinity (K_D~0.1nM) receptors and a majority (95%–98%) of low-affinity (K_D~10nM) receptors. The two site binding leads to concave-up type Scatchard plots (Schlessinger *et al*., 1986, 88). The structural identifications of different conformations of the EGFR extracellular regions suggested that the low affinity binding sites might represent receptors in the auto-inhibited conformation, whereas high affinity binding sites might be accounted for by receptors in the active, extended

configuration (Klein *et al*, 2003(6-11)). However, this hypothesis could not explain the concave-up character of the experimentally observed Scatchard plots. Furthermore, the data does not support the dimerization model, which assumes that receptors in the active conformation bind ligand with high affinity and form receptor-receptor dimers (Klein *et al.*, 2003). Ozcan *et al*., argued that the concave-up curvature typical of an EGF binding Scatchard plot for wild type EGFR can be simulated only by including in the model a saturable high affinity external site for receptor dimers. Additionally, they argued that the so called low affinity EGF-binding sites seen in studies of the wild-type EGFR represent the interconverting tethered and extended (dimerizing) form of the receptor extracellular domain inferred from structural studies (Ozcan *et al*., 2006). Further studies by Macdonald *et al*., proposed that the concave-up Scatchard plots can be explained by including negative cooperativity in the system. The results from Macdonald *et al*., studies indicated that the affinity of EGF binding to the second site on a dimer (2.9nM) is 15-fold less than the affinity of EGF for binding the first site on a dimer (190pM). All of these studies supported that intracellular domain is required to account for concave-up Scatchard plots although it is clear that the extended configuration of the EGFR binds EGF with higher affinity than does the unextended configuration (Klein *et al*., 2003). All the above mentioned studies were carried in cells.

In non-cellular studies using soluble extracellular EGFR, the binding experiments and structural data suggest that EGF can bind to the sEGFR in at least two separate types of binding events (Lemmon *et al*., 1997). However, the generated Scatchard plots were not of concave-up character (Klein *et al*., 2003). In previous Biacore studies using soluble

extracellular EGFR, the apparent K_D for EGF binding was reported to be 100-400nM (Wade *et al*., 2002, Iyer *et al*., 2007, Ferguson *et al*., 2003). Wade *et al*., reported that analysis of equilibrium binding data in Scatchard format for Biacore studies of EGF/sEGFR interaction gave an excellent linear fit (R=0.992) and indicated a K_D of 292nM but the curves did not fit ideally to a 1:1 Langmuirian model. A better fit was obtained with a model that comprised a small percentage of high affinity component indicating two independent binding events. The presence of high affinity was also suggested from Biacore solution competition data and fluorescence anisotropy experiments yielding biphasic Scatchard plots (Domagala *et al*., 2000).

Based on the previous Biacore studies, we designed our experiment for EGF/sEGFR interaction such that it closely imitates the physiologically active system of cell. We aimed to reproduce the literature binding kinetics and tried to explore further into the concepts of multiple binding events. A summary of the previous studies is given in Table 2.

Table 3.2-1 Experimental systems used for EGF/EGFR interaction

Reference	**Analyte**	**Ligand**	**Type of Study**	**Interaction type**	**Results (Scatchard Plot)**
Schlessinger *et al*., 1986, 88.	EGF	EGFR	In cells	Heterogeneous	Curvilinear
Domagala *et al*., 2002	hEGF	sEGFR	Biacore	Heterogeneous	Biphasic
Wade *et al*., 2000	sEGFR	mEGF	Biacore	Heterogeneous	Linear

3.3 Review of previous studies on Cetuximab binding to EGFR

Cetuximab is a chimeric monoclonal antibody of the immunoglobulin G1 (IgG1) class that is directed against the human epidermal growth factor receptor (EGFR) and binds to extracellular domain of the receptor with high specificity. Cetuximab has been developed jointly by Merck, KGaA, and ImClone Systems Incorporated/Bristol-Myers Squibb for the treatment of several types of human cancer that express EGFR, including colorectal cancer, squamous cell carcinoma of the head and neck, nasopharyngeal cancer, pancreatic cancer, ovarian cancer, and non-small cell lung cancer.

Biacore binding studies of Cetuximab Fab fragment with sEGFR have shown a simple 1:1 Langmuir binding with an apparent K_D of 2.3±0.5nM (Li *et al*., 2005). In this experiment, Cetuximab Fab fragment was immobilized and sEGFR was made to flow on the surface. In the studies carried over by Tikhomirov *et al*, sEGFR was immobilized on the sensor surface and Cetuximab was used as analyte. These studies reported binding in both high as well as low sEGFR surface density scenario. Low immobilization levels of sEGFR were used to represent monovalent binding conditions and high immobilization levels were used to show bivalent binding conditions. In studies carried over by Goldstein *et al*., an equilibrium dissociation constant of 0.2nM was reported using Biacore studies where sEGFR was amine coupled to the surface and Cetuximab was the analyte.

In our studies, we tried to imitate the system close to that in living cells, immobilizing sEGFR to the surface. Also, we used two different surface densities (one higher and the other lower) to compare the binding of Cetuximab to explain avidity effects on the surface.

We reported the kinetic dissociation rate constant of Cetuximab/sEGFR interaction for the first time in our studies.

3.4 Considerations for Biacore experiments

The following constraints must be considered while designing a binding experiment on Biacore and analyzing the data obtained.

3.4.1 Mass Transport

Biacore has a flow cell which consists of a sensor surface on top of which one of the interactants (ligand) is immobilized. The other interactant (analyte) is passed over the flow cell and binding is determined by the optical system.

In Biacore, analyte is transported by diffusion and flow to the sensor surface, where it reacts with the immobilized receptor (Myszka *et al*., 1998). Under laminar flow conditions used in Biacore, the rate of transport of the analyte to the surface is proportional to the cube root of the flow rate, and is also influenced by the dimensions of the flow cell and the diffusion properties of the analyte (Jason-Moller *et al*., 2006). The interaction is reaction limited when the transport of analyte to the surface is much faster than the rate of analyte association with the ligand. The observed binding here will be determined by the kinetic rate constants (Jason-Moller *et al*., 2006). On contrary, the binding is mass transport limited when the rate of analyte transport to the surface is slower than the reaction rate of

analyte to the ligand. This may lead to partial or no kinetic information of the binding. Optimal assay conditions have to be determined to minimize mass transport limitations to get reliable kinetic information.

From the Biacore studies, it was determined that mass transport limitations majorly depend on two factors: surface density of the ligand and flow rate of the analyte to the surface. As the receptor density on the sensor surface is increased, the binding reaction at the surface speeds up, and the binding kinetics become transport limited (Myszka *et al*., 1998). High flow rates help in delivering consistent amount of sample to the surface. Hence, the kinetics is best studied under conditions of high flow rates and low surface binding capacity. In practice, this translates to using ligand densities that result in a maximum analyte binding response no greater than 50 to 150RU and flow rates greater than 30µl/min (Jason-Moller *et al*., 2006).

3.4.2 Analyte Concentrations

Analyte concentrations play an important role in accurately determining the equilibrium or association rate constants correctly. The surface is said to be 50% saturated with analyte at the concentration equal to equilibrium dissociation constant K_D. It is recommended that the concentrations cover a full range of "binding curves" showing barely binding of analyte to "saturation binding". Reliable detailed kinetic analysis requires data from four to six analyte concentrations, spanning the range of 0.1 to 10 times K_D (Jason-Moller *et al*., 2006). Analytes should be in the same buffer as the continuous

flow buffer to minimize bulk refractive index differences that can lead to low signal-to-noise ratios. This is often most easily achieved through dilution of a concentrated analyte stock into running buffer. Kinetic assays should include a series of start-up cycles using buffer as analyte to equilibrate the surface as well as cycles with zero concentration of analytes as part of the concentration series for the purposes of reference subtraction (Myszka *et al*., 1999). Although it is not necessary to reach equilibrium, it is recommended that the association times used be sufficient for at least one analyte concentration to reach steady state. To accurately determine dissociation rate constants, a measurable decrease in signal should occur during the dissociation period (Jason-Moller *et al*., 2006).

CHAPTER 4 Experimental Background

4.1 Surface Plasmon Resonanace

Surface Plasmon Resonance (SPR) is a relatively new technique used to measure biomolecular interactions in real time. This is done in a label free environment, while one of the interactants is immobilized on to the sensor surface and the other is passed over the surface. The association and dissociation of the interactants are measured in resonance units (RU) and plotted as a Sensogram.

4.1.1 Principle of SPR

SPR is a phenomenon that occurs in thin conducting films at an interface between media of different refractive indices. In the Biacore instrument, the conducting film is thin layer of gold, and the media are the glass of the sensor chip and the sample solution. When a plane polarized light is incident onto the reflecting interface, under conditions of total internal reflection, an electric field intensity known as an evanescent wave is generated. This evanescent wave is exponentially detenuating with distance from the surface. At a specific incident angle and energy (wavelength), the incident light excites plasmons in the

gold film and SPR is seen as a drop in the intensity of the reflected light due to the resonance energy transfer between evanescent wave and surface plasmons. The resonance conditions are influenced by the material absorbed onto the thin metal film and the SPR signal depends on the refractive index of solutions in contact with the surface.

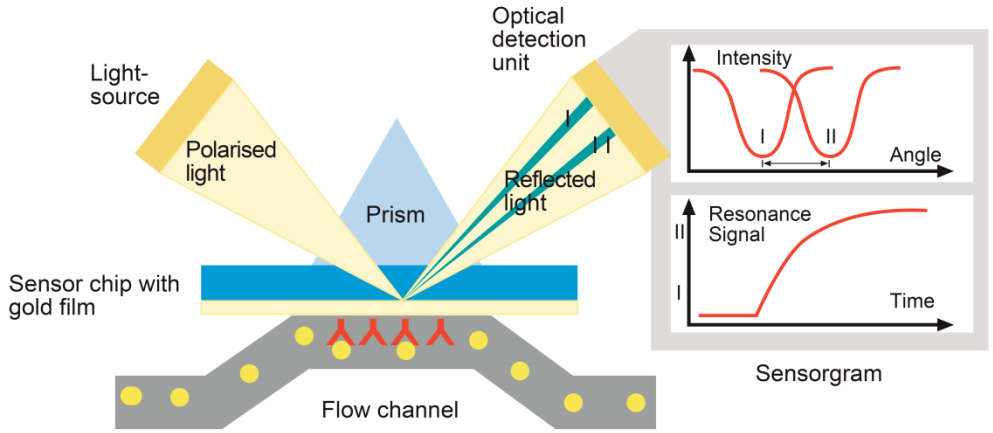

Figure 4.1-1 Demonstration of Surface Plasmon Resonance. The binding of analyte shifts the SPR angle from position I to II.

In Biacore, the immobilized molecule on the surface is called the ligand and the molecule that flows over the surface is called the analyte. As analyte binds to the ligand, there is accumulation of protein on the surface which results in an increase in the refractive index. This change in refractive index is measured in real time and plotted as response or resonance units (RU) versus time. This plot is called a Sensogram and gives information on kinetics, affinity, binding specificity and concentration profiles. The technology allows determination of these parameters with analytes ranging in size from about 150 to 10^6 g/mole (Dalton).

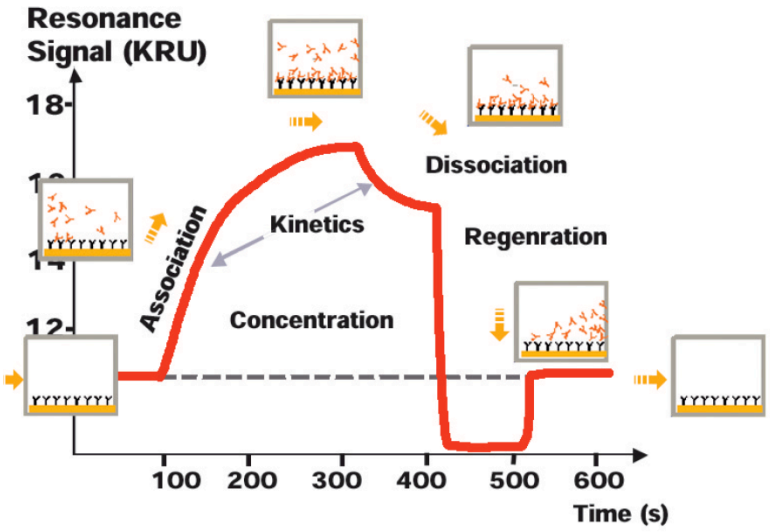

Figure 4.1-2 Sensogram

4.2 Instrumentation

The Biacore instrument is composed of three main units: the optical system, a liquid handling system and the biosensor chip. The instrument is connected to a PC running Biacore, where the results are presented in real time as a Sensogram.

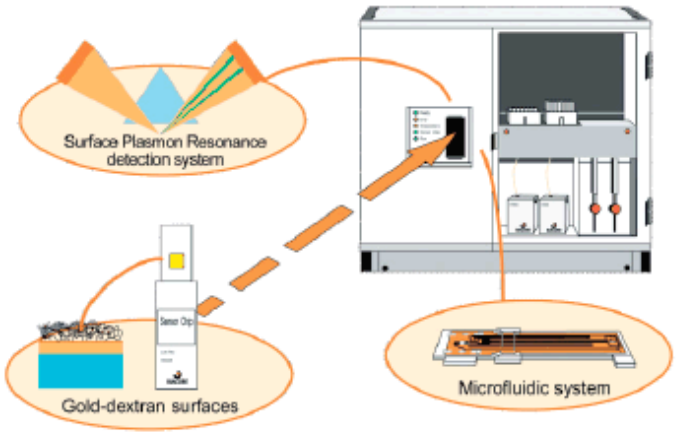

Figure 4.2-1 Three corner stones of Biacore technology

4.2.1 Optical System

The optical system is responsible for generation and detection of the SPR signal. It detects all surface changes in refractive index as the mass changes in the aqueous layer close to the sensor chip surface. The optical unit has a glass prism on to which the glass slide of the sensor chip is kept in contact. A good optical coupling between the prism and sensor chip is ensured by a silicone opto-interface. Light is focused through the prism on to the sensor chip surface from a near-infrared light-emitting diode (LED), giving a fixed range of incident light angles. Light reflected from the sensor chip is monitored by a linear array of light-sensitive diodes covering the range of incident light angles. By using a wedge of incident light and a fixed array of detectors, the SPR angle is monitored accurately in real time (Bia technology handbook).

4.2.2 Liquid Handling System

The liquid handling system with precision pumps and an integrated microfluidic cartridge (IFC) in Biacore maintains a constant flow of sample and buffer over the sensor chip surface. The autosampler in the system helps in transferring, diluting and mixing samples, injecting samples into the IFC. One of the two pumps maintains a continuous flow of liquid through the detector flow cell while the other assists in the working of the autosampler. Samples are transferred through a needle from the autosampler into the IFC, which connects with the flow cells. The microfluidic system allows single or multichannel analysis in up to four flow cells. One of the flow cells can be used as a true reference during sample injection, allowing blank-subtracted data to be presented on screen during analysis. The data obtained thus enhances the signal quality and maximizes the resolution.

4.2.3 Sensor Chip

The Biosensor chip is a glass slide coated with thin layer of gold, creating the physical conditions required for generating an SPR signal. This forms the base for the attachment of a range of specialized surfaces designed to optimize the binding of various molecules (Malmqvist *et al*., 1999). On most sensor chips, a matrix of carboxymethylated dextran layer is covalently attached to the gold surface forming a surface layer of approximately 25-100nm thick. The dextran layer maintains a hydrophilic environment suitable for a variety of protein interactions and helps in covalent immobilization of bio

molecules. The sensor chip can be divided into four flow cells and each flow cell can be used independently (Wei *et al*., 2004).

Sensor chip CM5 is the most commonly used versatile chip which supports immobilization of wide range of ligands from small organic molecules to proteins, nucleic acids and carbohydrates. Molecules are covalently coupled to the sensor surface via amine, thiol, aldehyde or carboxyl groups. The chip is mounted on a plastic support frame that is protected by a plastic cassette.

Some other sensor chips include sensor chip SA which is used to capture biotinylated peptides, proteins and DNA, sensor chip NTA which is used to capture ligands via metal chelation, sensor chip HPA which is for membrane biochemistry and the study of membrane associated receptors.

4.3 Immobilization

Biomolecules for interaction can be attached to the sensor surface using two different approaches: Covalent immobilization and Capturing methods.

4.3.1 Covalent Immobilization

Covalent immobilization is the most commonly used approach for attaching the ligand to the dextran matrix on the sensor surface. The various covalent immobilization chemistries used are: Amine coupling, which couples amine groups on the ligand to the

carboxyl groups on the dextran matrix; Thiol coupling, which is used when there can be thiol-disulfide exchange between thiol groups and active disulfides introduced on either the ligand or the surface matrix; Aldehyde coupling, which is used when there can be reaction between hydrazine or carbohydrazide groups introduced on the surface and aldehyde groups obtained by oxidation of carbohydrates in the ligand.

Covalent immobilization may have limited stability under certain conditions, although it generally results in stable attachment of ligand to the surface under the buffer conditions normally used for interaction analysis and regeneration. Also, it might involve chemical modification of the ligand which can potentially affect the analyte-binding activity. But most ligands can be immobilized without losing activity by one or the other chemistry.

4.3.2 Capturing Approaches

Capturing is the best alternative approach when covalent immobilization results in loss of ligand activity or is unsuitable for other reasons. The capturing molecule is covalently attached to the matrix to which the ligand is immobilized. The ligand interaction with the capturing molecule should have a high affinity so that the ligand does not detach from the molecule during analysis. Capturing approach does not introduce conformational changes in the ligand and the orientation of ligand is in a specific direction. But capturing approaches consume more ligand when fresh material has to be captured for each analysis since some ligand dissociates from the surface during regeneration. The

common capturing approaches are streptavidin- or avidin-biotin capture, antibody-based capture, and capture of tagged proteins.

4.4 Amine Coupling

Amine coupling is used to immobilize molecules which contain amine groups. This is the most generally used coupling chemistry since most of the biomolecules contain amine groups. Firstly, carboxyl groups on dextran surface are activated by passing a mixture of EDC and NHS to yield reactive ester groups. Ligand is then passed over the surface where the esters spontaneously react with primary amine groups or other nucleophilic groups on the ligand and link it covalently to the dextran matrix. Injection of ethanolamine after ligand immobilization deactivates the remaining active ester groups on the surface and removes non-covalently bound ligand. Typical Sensogram of amine coupling is shown in Figure 4.4-1.

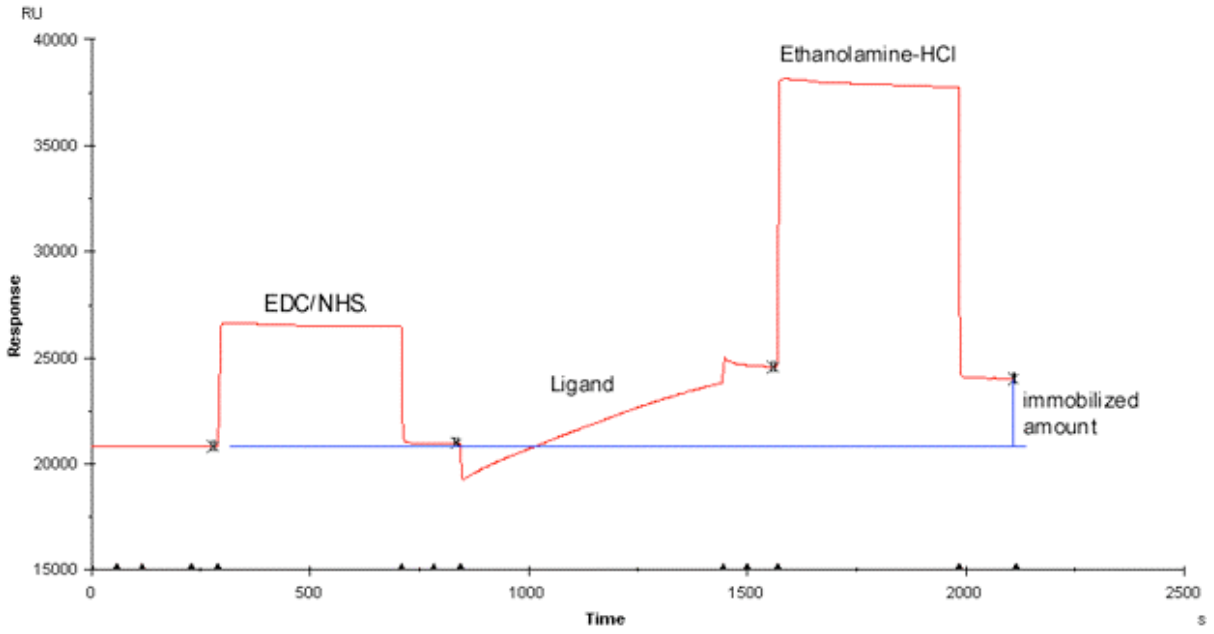

Figure 4.4-1 Illustrative graph of Amine coupling

4.5 Kinetics

Biacore has several applications which include defining the characteristics of proteins in terms of their specificity of interaction with other molecules, their affinity of binding to other molecules, and the rates at which they interact (Karlsson *et al*., 2006). Biacore system also provides accurate concentration measurements.

The application of interest in this project is kinetics since it provides dynamic binding information as in actual cellular processes. As the analyte binds to the ligand, there is increase in mass at the sensor surface which is recorded as association phase and as analyte dissociates from the ligand, the decrease in mass at the surface is recorded as dissociation phase in the Sensogram. When the rate of association and rate of dissociation are equal,

there is equilibrium established, which is marked by steady state in the Sensogram. For 1:1 Langmuir interaction, the dissociation constant, K_D, is the ratio of kinetic rate constants, k_d/k_a. A lower value of K_D represents greater affinity of the ligand-analyte interaction.

For kinetic applications, the ligand immobilized has to be low to avoid mass transport effects. For best kinetic results, the maximum analyte binding capacity, R_{max} is in the range 50-150RU. The level of ligand to be immobilized is calculated using the manufacture's recommended formula, $R_{max} = (MW_a/MW_L) * R_L * Sm$. The concentration range of analyte is $0.1K_D$ to $10K_D$.

4.6 Regeneration Scouting

The analyte has to be completely dissociated from the ligand to start a new analysis cycle. The extent of dissociation in running buffer varies with the type of analyte and how strong it binds to the ligand. A regeneration solution has to be passed over the surface to remove the bound analyte from the ligand of the surface. The conditions for regeneration have to be such that the ligand over the surface is not damaged or does not lose activity. Regeneration scouting method helps in finding suitable regeneration conditions for the ligand, which is determined for our system as described in Chapter 5 below.

CHAPTER 5 Materials and Methods

5.1 Materials

SPR measurements are performed using Biacore 3000 system. The antibodies, buffers and reagents used for the experiments are listed below.

5.1.1 Antibodies

The receptor sEGFR was used as the ligand and the growth factor EGF and the cancer drug Cetuximab were used as analytes to study the interaction. Also the binding interaction of sEGFR with a peptide (P-13), designed specifically for this target was also studied. The human recombinant sEGFR was purchased from Research Diagonostic Inc and EGF was purchased from Sigma. Another 100mg/ml of EGF was purchased from BD Biosciences for additional experiments. The Cetuximab was purchased from the pharmacy of Massey Cancer Center. The P-13 was synthesized using CEM® Automated Microwave Peptide Synthesizer.

5.1.2 Buffers and Stock Solutions

The buffers and other reagents used for Biacore experiments are listed in the Table 5.1-1 below. All solutions were made up of DI H_2O from Millipore.

Table 5.1-1 Buffers and Stock solutions for Biacore

Name of the Solutions	Components
HBS-EP	Aqueous buffer containing 0.01 M HEPES pH 7.4, 0.15 M NaCl, with 3mM EDTA and 0.005% Surfactant P20.
Amine Coupling Solutions	EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride); and NHS (N-hydroxysuccinimide); and Ethanolamine 1M.
Regenerations Solutions	Glycine 2.0; or Glycine 2.5; or NaOH 10mM; or NaOH 50mM;
Ligand stock solution	10mM NaAc pH 5.2±0.1
BIAnormalizing solution	70% glycerol
BIAdesorb solution 1	0.5% sodium dodecyl sulphate (SDS)
BIAdesorb solution 2	50mM glycine-NaOH pH 9.5
BIAdisinfectant solution	Sodium hypochlorote with 8-12% active chlorine

5.1.3 Reagents and Stock Solutions for Peptide Synthesis

The reagents and other solutions used for peptide synthesis and purification are listed in the Tables 5.1-2, 5.1-3, 5.1-4 below. All amino acid solutions are prepared in DFM.

Table 5.1-2 Reagents and Stock Solutions for peptide synthesis.

Name of the Solutions	Components
Main Wash	DFM (N, N-Dimethylformamide); and DCM (Dichloromethane).
Activator	8.53g HBTU dissolved in 45ml DMF.
Activator Base	A solution of 17.4ml DIEA (N, N-Diisopropylethylamine) mixed with 32.6ml of NMP (1-Methyl-2-Pyrrolidone).
Deprotect	A solution of 100ml piperidine, 400ml DFM (N, N-

	Dimethylformamide) and 6.75g HoBt.
Capping mix	20% of Acetic anhydride in DMF (4ml of Acetic anhydride in 16ml DMF).
Cleaving mix	A solution of 250µl DODT (3, 6-Dioxa-1, 8-octane-dithiol), 250µl TIS (Triisopropylsilane), 250µl H_2O mixed in 9.25µl of TFA (Trifluoroacetic acid).
Peptide precipitate mix	Diethyl ether (or cold ether).

Table 5.1-3 Reagents and buffers used for protein purification (HPLC).

Name of the Solutions	Components
B.conc	0.1% TFA in MeCN (Acetonitrile).
A. conc	0.1% TFA in DI H_2O.

Table 5.1-4 Solutions used for MALDI Mass Spec.

Name of the Solutions	Components
Matrix	500µl of 0.1% TFA mixed in the solution of 10mg crystallized CHCA and 500ul Acetonitrile.

5.2 Equipment

5.2.1 Biacore 3000

The binding studies of the proteins and antibodies were carried out using Biacore 3000 instrument from GE Healthcare. The experiments were carried out at 25ºC with a data collection rate of 1Hz. The running methods were created using the Biacore 3000 control software 3.0. The Sensograms were evaluated using BIAevaluation 3.0 software provided by the manufacturer. Maintenance of the instrument was performed according to the supplier's instructions.

5.2.2 CEM Microwave Peptide Synthesizer

Liberty-Automated microwave peptide synthesizer from CEM Corporation was used to make the peptide P-13. This instrument uses microwave energy to drive biochemical reactions resulting in higher purity peptides made 10 times faster than by conventional methods. All amino acid bottles and reagent bottles were cleaned and back flushed before starting the synthesis.

5.2.3 Other Instruments

MALDI Mass Spectrometer was used to check the mass of the peptide synthesized. The data was collected and analyzed using the Mass Lynx software provided by the manufacturer. HPLC (High Performance Liquid Chromatography) was used to purify the peptide. The software used for analysis of data was LC solutions provided by the manufacturer. The Eppendorf Microcentrifuge was used to filter the peptide samples for HPLC purification. The samples were filled in microcentrifuge vials and were centrifuged at 13.5rpm speed for 1min. Rotary Evaporator from Fisher Scientific was used to remove solvents from the peptide solution after purification.

5.3 Peptide Synthesis

The peptide synthesis was carried out using Automated Microwave Peptide Synthesizer from CEM Corporation. All amino acids and reagents needed for synthesis were freshly prepared before the start of the experiment.

5.3.1 Synthesis of P-13

The amino acid sequence was input to the instrument software which subsequently calculates the amounts of reagents required for the synthesis. The amino acids were prepared dissolving the calculated amounts of respective samples in the given volumes of DMF as recommended by the CEM program. The activator used was HBTU dissolved in DMF and the activator base was a solution of DIEA and NMP. A solution of piperidine, DMF and HOBt was used as deprotect mix and 20% Acetic acid in DMF was used as a capping mix for the synthesis. The resin used was Fmoc-PAL-PEG. The synthesis ran for 9hrs for this 13-residue peptide.

5.3.2 Washing and Cleaving the Peptide

The resin having the peptide was washed with DCM after filtering it out from DFM. The excess DCM was filtered out and the resin with peptide was subject to cleavage in a solution of DODT, TSI, H_2O and TFA. The resinous solution was filtered and the filtrate was mixed with cold ether which was centrifuged to give out a precipitate and stored at -20°C as peptide.

5.3.3 Purification of the peptide

High Performance Liquid Chromatography (HPLC) was used to purify the peptide from other salts after cleaving it from the resin. A peptide solution of 20mg/ml concentration was made by dissolving a pinch of the peptide in 0.1%TFA & H_2O. An analytical procedure was carried out on HPLC to determine the gradient of mobile phase component "B" for peptide extraction. After obtaining the gradient, the separation was carried out on a large scale and the purified solution was tested on Mass Spec to reconfirm the presence of the peptide by checking the mass of the obtained sample. The mass acquisition range specified was from 800-3000Dalton. A rotary evaporator was used to remove solvents by evaporation from the peptide solution. The obtained peptide is weighed using a mass balance and stored at -20°C for Biacore applications.

5.3.4 Concentration of the peptide

Biophotometer was used to determine the concentration of the peptide at A_{280nm}, since using a mass balance has not proven to be an accurate way to get the weight of the peptide; the peptide mass synthesized (~mg) is too small and even a minute quantity of water or other substances sticking to the vial would affect the accuracy of weight shown. The peptide was dissolved in 1ml DI H_2O and 3µl of H_2O was used as reference blank. Peptide volume of 3µl was given as sample and the absorbance value was displayed on the screen. The concentration of the peptide is then calculated using the equation $A=ebc$ (Beer-

Lambert law), where, A is the absorbance of the sample, b is the path length, c is the concentration in mol/l and e is the extinction coefficient of the peptide which is calculated as referenced in Kibbe WA *et al*., 2007. Multiplying the calculated concentration with the molecular weight of the peptide (1688g/mol) gives the concentration of the peptide in g/l which can be converted to mg/ml.

5.4 Surface Preparation

A research grade CM5 sensor chip from Biacore was used to immobilize ligand on flow cell 2 or 4 using flow cell 1 or 3 as reference accordingly. The bare chip was conditioned by passing 50mM NaOH for five cycles for 1min through all the flow cells. This procedure removes all loosely bound dextran material from the surface. Then the chip was normalized according to the supplier's instructions. This gives maximum sensitivity of the chip. The sensor surface and sample blocks were maintained at 25°C.

5.4.1 Preparation of sEGFR as ligand

The human recombinant soluble EGFR (purchased from Research Diagnostic Inc) was used as the ligand. The ligand was diluted with 10mM sodium acetate buffer to a concentration of 25µg/ml. The ligand was diluted to give a solution of pH 5.2±0.1 which falls in the required range (3.5 and isoelectric point of the ligand). The carboxymethylated dextran on the sensor chip is negatively charged above the pH 3.5 and electrostatic

attraction provides an efficient means for concentrating positively charged ligands on the surface. Hence, the ligand was prepared in 10mM NaAc (pH 5.2±0.1) to give a final concentration of 25µg/ml (Iyer *et al*., 2007). This concentration was used for the binding studies of all the three analytes- EGF, Cetuximab and peptide P-13.

5.4.2 Immobilizing sEGFR on CM5

The amine coupling kit from Biacore was used for the procedure. The EDC and NHS solutions were prepared according to the manufacturer's instructions. The ligand level to be immobilized was determined using the formula as discussed in Chapter 4,

$$R_{max} = (analyte\ MW/ligand\ MW) * Stoichiometry * R_{L.}$$

The binding capacity of the surface depends on the ligand immobilization level. Here R_L is the immobilization level of the ligand which determines the maximum response, R_{max}. For the experiment, the R_{max} has been considered to be in the range 50-150RU. Here, the molecular weight of sEGFR is 82kD. The molecular weights of EGF, Cetuximab and the peptide P-13 are 6kD, 152kD and 1.688kD respectively. Fitting in the values of molecular weights for the given proteins in the equation, a suitable range for the immobilization level of sEGFR was determined for all three analytes. Considering only 60% of the ligand is active on the surface, the immobilization level is scaled up accordingly. Hence an immobilization levels in the range 2500-5500RU for EGF, about 100-400RU for Cetuximab and about 2500-4000RU for P-13 were achieved.

The sensor chip has four flow cells out of which flow cells 1 or 3 were used as reference cells and immobilization was done on flow cells 2 or 4. Immobilization was carried out in two ways: Using 'Aim for Immobilized level' wizard or using 'Specify flow rate and injection time' wizard. The first few experiments were carried out using 'Aim for immobilized level' wizard with target RU set to 2500RU for EGF-sEGFR interaction. The later were carried out specifying flow rate and injection time. The flow rate and injection time was specified for all Cetuximab-sEGFR and P-13-sEGFR experiments. The flow rate and injection time were adjusted such that the target RU (as per calculations above) was reached.

5.5 Kinetic Analysis of protein-protein or peptide-protein interactions

The kinetic studies of sEGFR-EGF, sEGFR-Cetuximab and sEGFR-P-13 were carried out using the kinetic analysis wizard. HBS-EP was used as the running buffer.

5.5.1 EGF Interaction

EGF was serially diluted in sterile HBS-EP buffer with the dilution factor of 2 between each concentration. The concentration range used was 1000nM to 10nM and a zero concentration sample was included for reference subtraction. The analyte concentrations were adjusted such that the lowest concentration barely shows binding to the ligand and highest concentration reaches saturation. The wizard template had the run

order sorted from low analyte concentration to high analyte concentration. The samples were injected over the surface at a flow rate of 30µl/min with a 3min association pulse and 15min of dissociation, without the need for a regeneration step (Iyer et al., 2007).

5.5.2 Cetuximab Interaction

The drug Cetuximab, also called C225 was diluted in sterile HBS-EP buffer to a series of concentrations. The analyte concentrations for analysis ranged from 0.2nM to 30nM with a dilution factor of 2 between each concentration. Also, a high concentration sample of 100nM and a zero concentration (plain buffer) sample were included for analysis. The samples were run from low to high order at a flow rate of 30µl/min with 2min association and 10min dissociation. In order to find the optimal conditions for dissociation of the ligand-analyte complex, a regeneration scouting method was run with glycine as regeneration solution at three different pH values. The solutions were tested from mild to harsh conditions. The solutions used were glycine pH 3.0, glycine pH 2.5, glycine pH 2.0. It was observed that the regeneration was best when glycine pH 2.5 was used as regeneration solution. In this case, the ligand surface returned to within 1% of its initial RU value. Hence, the surface was regenerated with a single injection of 10mM glycine (PH 2.0) for 1 min after dissociation and a stabilization time of 1min was included.

5.5.3 Peptide Interaction

The peptide P-13 was initially diluted using HBS-EP buffer to higher concentrations and tested on the sensor chip immobilized with sEGFR for binding. The concentrations were increased in the next subsequent runs when significant binding was not observed. The regenerations conditions were determined by performing regeneration scouting with regenerations solutions glycine at pH 3.0, pH 2.5, pH 2.0, pH 1.5 and 10mM NaOH. The solutions were tested from mild to harsh conditions. After determining the concentration of analyte which gives good binding to the chip and a suitable regenerations solution which dissociates the complex, analyte dilutions were carried out with a minimum concentration of 200uM and maximum concentration of 1000µM. A zero concentration sample was included for reference subtraction. Kinetic analysis wizard was run with the series of concentrations with an association time of 5mins and dissociation time of 15mins at flow rates 30µl/min. The surface was regenerated with a single injection of 10mM NaOH for 30seconds after dissociation and a stabilization time of 1min was included.

CHAPTER 6 Results and Discussion

The results of binding kinetics studies of antibodies EGF, Cetuximab and P-13 with sEGFR carried on Biacore are presented in this chapter along with mass and purification data of the peptide P-13. Comparisons to previously reported results are given for the EGF-sEGFR system and Cetuximab-sEGFR system. We demonstrate that our Biacore results for the EGF-sEGFR system are consistent with cell culture results.

6.1 Synthesis of P-13

6.1.1 Calculating the mass of P-13

The mass of the peptide was calculated using peptide mass calculator v3.2 (Jef Rozenski (1999)) available on web by inputting the peptide sequence. The peptide mass was calculated considering that the N-terminal of the peptide was acylated and C-terminal was an amide bond. The calculated peptide mass was 1688.737 Dalton.

6.1.2 Determining P-13 mass using MALDI Mass Spec

MALDI Mass Spectrometer was used in molecular weight confirmation of the peptide P-13. The peptide was tested on MALDI before and after purification. Samples to be analyzed were prepared at ratio 2:8 peptide digest to matrix (crystallized α-Cyano-4-hydroxy-cinnamic acid, CHCA), and subsequently dried on a stainless steel MALDI plate. The mass data of the P-13 before purification (data not shown) and mass data after purification of P-13 using HPLC was consistent and confirms the presence of the peptide P-13. The mass data after purification is shown in Figure 6.1-1.

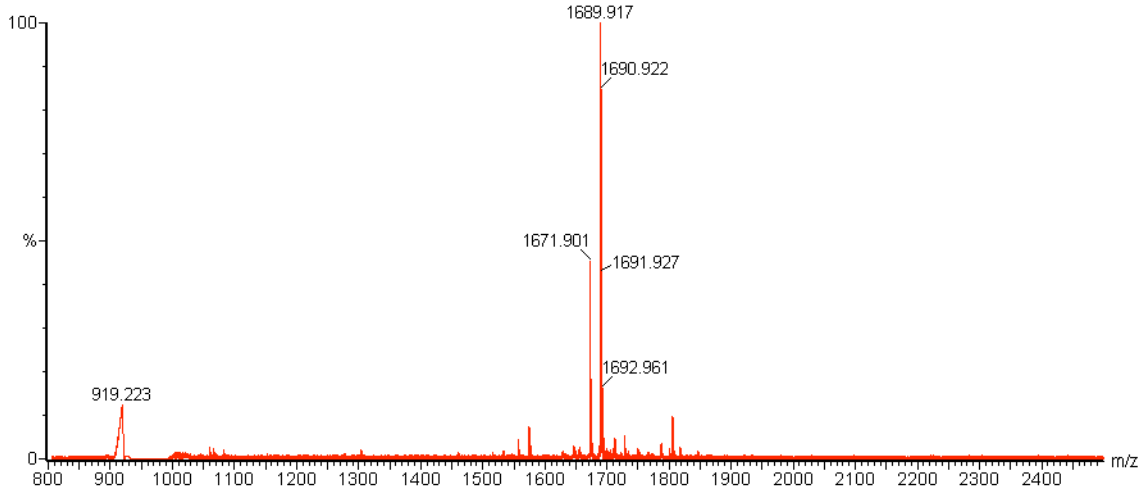

Figure 6.1-1 Data from Mass Spec for purified P-13.

The purified peptide from HPLC was collected in a vial and the solvents were evaporated using a rotary evaporator. The weight of the empty vial was recorded and the weight of the vial after rotary evaporation was recorded. The weight of the actual peptide was determined by carrying subtraction between the two weights obtained. One ml of DI H_2O was added to the known weight of the peptide formed and Mass Spec was run on the

sample. Also, the sample was tested for concentration on Biophotometer since mass balance can show drastic variations from the actual weight even with a minute quantity of other solvent present in the sample. The data of the Mass Spec is shown in the Figure 6.1-2. This confirms the presence of the peptide after rotary evaporation.

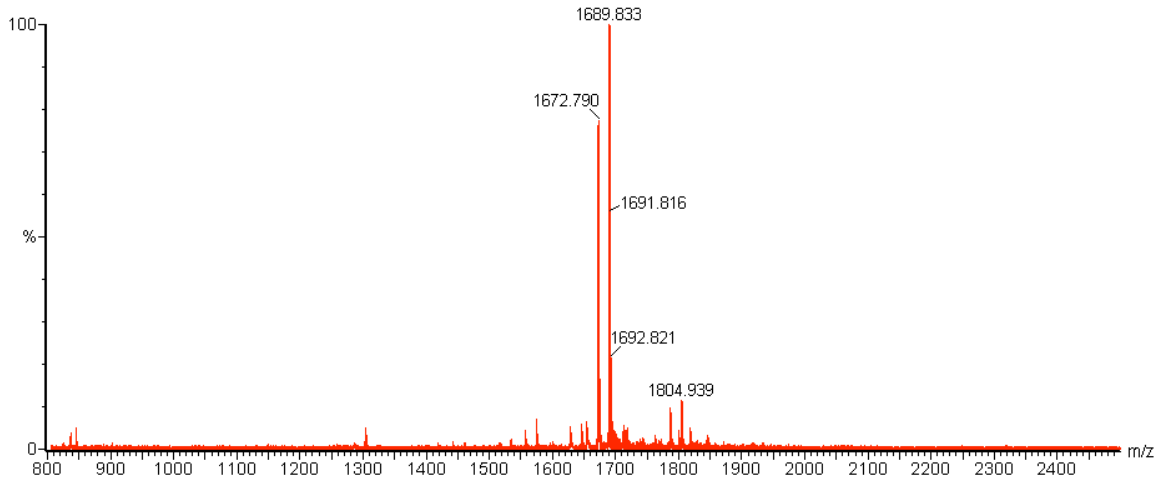

Figure 6.1-2 Data from Mass Spec for purified P-13 after solvent evaporation.

6.1.3 Purification of P-13

The peptide purification was carried on HPLC as described in Chapter 5. The peptide was recovered by gradient elution using a linear 50min gradient at a flow rate of 0.44ml/min. Detection was by absorbance at 280nm. Fractions were collected and analyzed using Mass Spectrometer for peptide mass. The fraction between 8-8.5min was identified to contain the peptide. The purification was then carried out on a large scale at a flow rate

of 10ml/min and the peptide was collected between 18-19mins. The peptide peak on HPLC is shown in Figure 6.1-3.

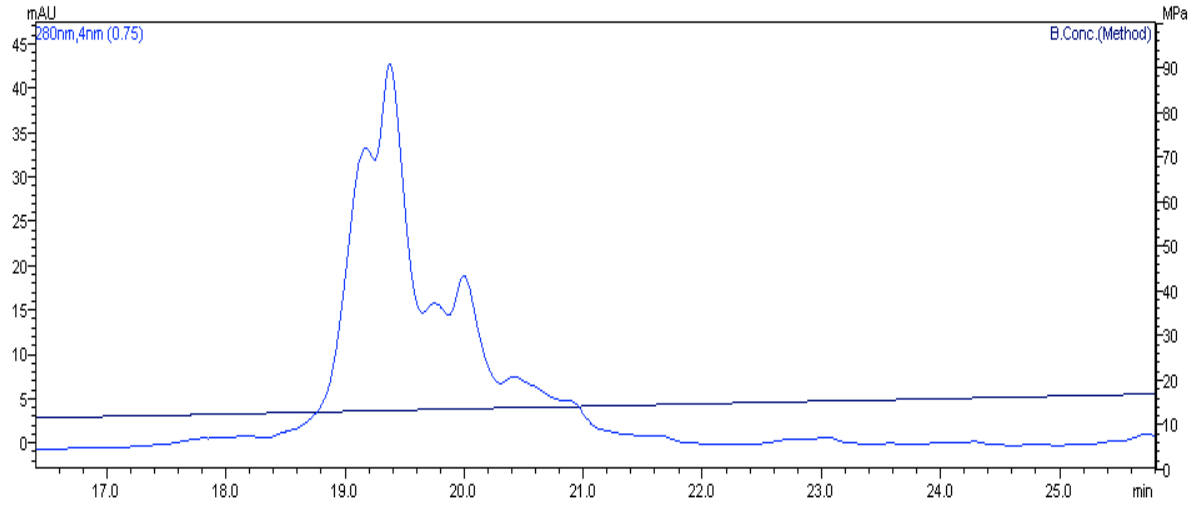

Figure 6.1-3 Chromatogram obtained from purification of P-13 using HPLC. The fragment was eluted between 18.5-19.6min, detected at wavelength 280nm.

6.2 Immobilization of sEGFR on CM5 sensor chip

The immobilization of sEGFR on CM5 sensor chip was done using amine coupling as described in the previous chapter. The immobilization levels (in RU) varied with analyte used for analysis.

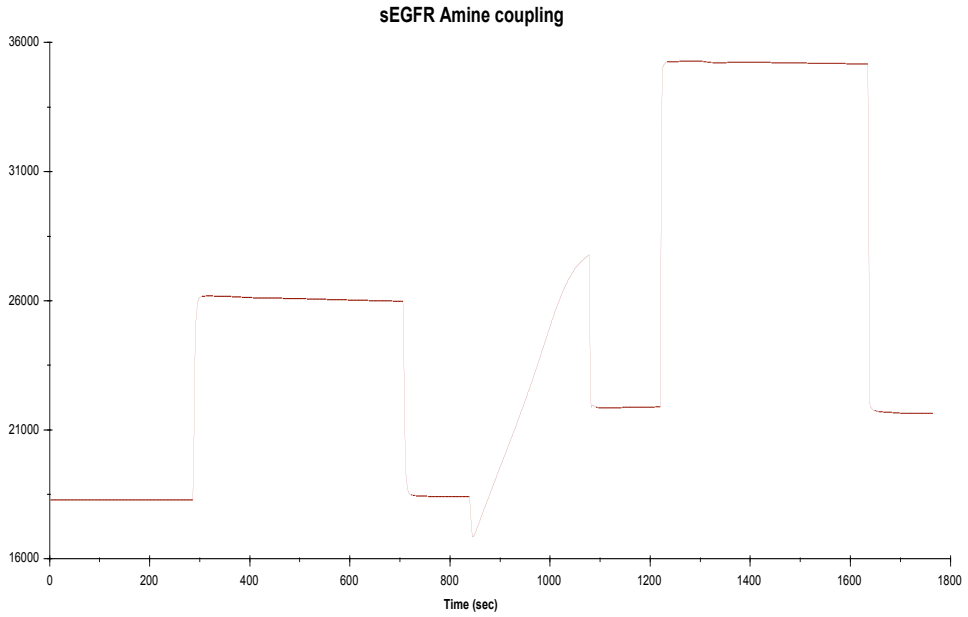

Figure 6.2-1 Immobilization of sEGFR using amine coupling. Surface is activated with EDC/NHS mixture before the ligand is injected to the surface and ethanolamine is used to deactivate the surface after ligand binding.

The sEGFR immobilization for EGF interaction studies was between 3000-5500RU. Cetuximab interaction studies were carried out with sEGFR immobilization level between 100-400RU. The immobilization level for peptide P-13 studies was between 2500-4000RU, each calculated according to the R_{max} equation as described in Chapter 4. A representative sEGFR amine coupling Sensogram is shown in Figure 6.2-1.

6.3 Kinetic analysis

The data obtained from binding studies of sEGFR with antibodies EGF, Cetuximab and P-13 are presented here.

6.3.1 Binding studies with EGF

The sEGFR ligand was immobilized on flow cell 2 using flow cell 1 as reference. For kinetic analysis, varying concentrations of EGF were passed over the sensor surface in order of increasing slope from the lowest concentration to the highest concentration. The running buffer was HBS-EP and the injected concentrations of EGF were 1000 (the curve with the highest RU), 500, 250, 125, 60, 30, 15 and 10nM. Data were analysed at the above mentioned concentrations of EGF with several densities of sEGFR (3357, 3700, 5500RU) immobilized on the surface. The representative Sensograms of the evaluated data are presented in Figure 6.3-1 below.

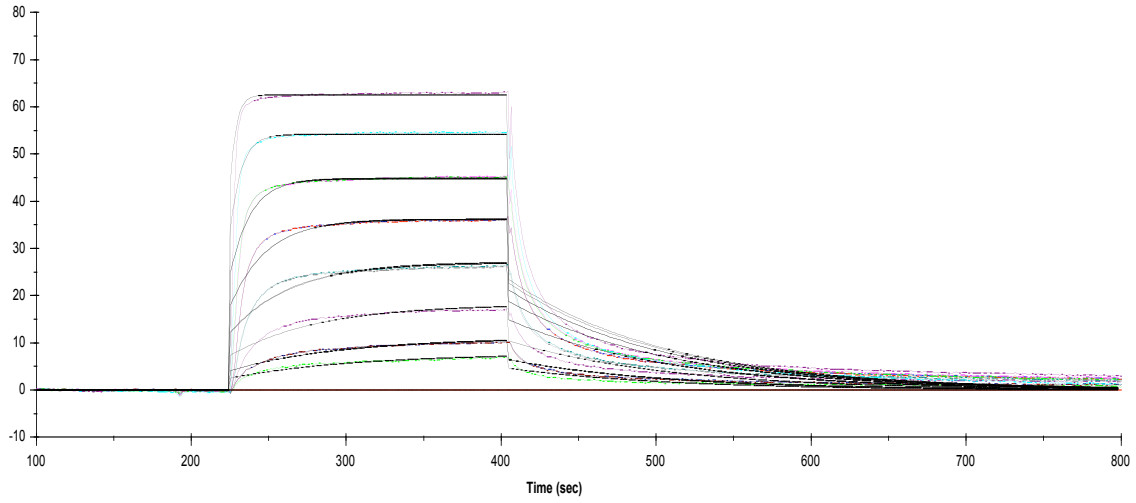

Figure 6.3-1a Kinetic analysis of EGF with sEGFR: Data fitted using 1:1 Langmuir binding model. Various concentrations of EGF were injected at 30µl/min over 3357RU immobilized sEGFR and reference surface (activated and deactivated by EDC/NHS and ethanolamine). Curves shown are reference subtracted.

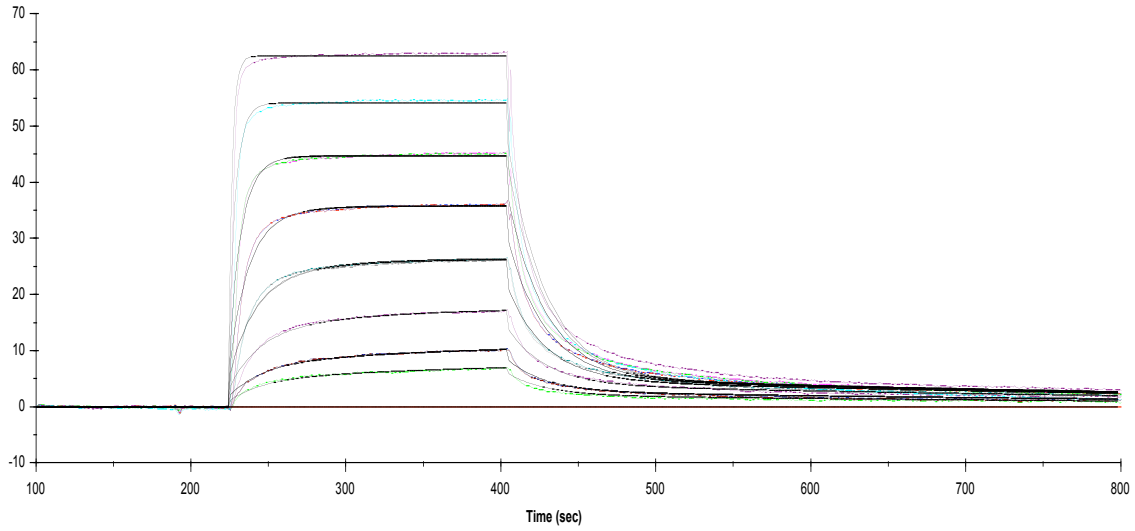

Figure 6.3-1b Kinetic analysis of EGF with sEGFR: Data fitted using heterogeneous ligand parallel interactions model. The data used is same as in Figure 6.3-1a.

The curves exhibit fast on and off rates and reach equilibrium binding rapidly. Both rate and equilibrium binding constants were determined fitting data using simultaneous k_a/k_d model (data fitted globally) as well as carrying over analysis of equilibrium binding data in Scatchard format.

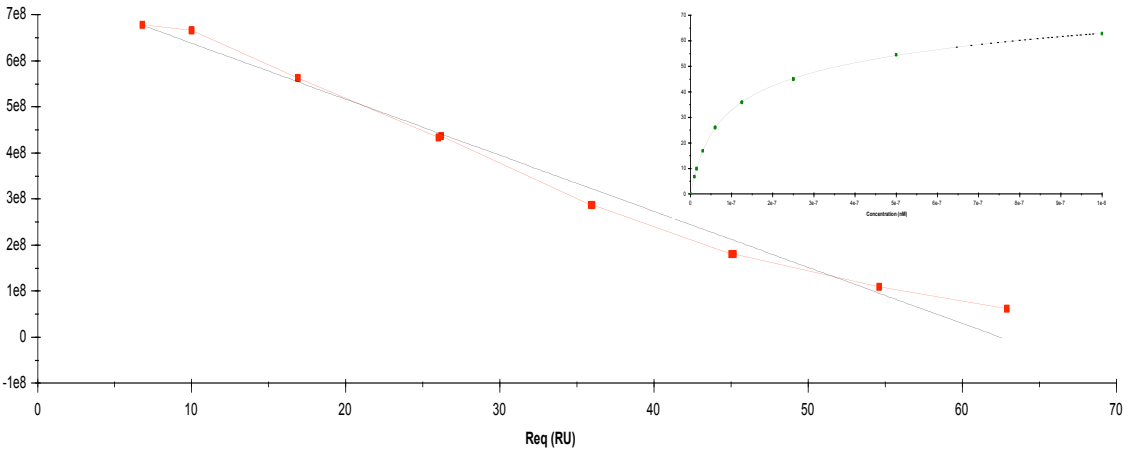

Figure 6.3-2 Scatchard plot of EGF binding to sEGFR. Steady state binding data was obtained by plotting R_{eq} against concentration (shown in the right upper corner). R_{eq}/Conc was calculated and plotted against concentration for Scatchard plot.

The Scatchard plot of equilibrium binding data of EGF with the receptor sEGFR is shown in Figure 6.3-2. The data seem to show deviations from linearity which indicates that there are more than one independent binding events occurring.

The x-intercept in the Scatchard plot of Biacore data represents the maximum analyte binding capacity R_{max} (~65 RU). For the immobilization of 3357RU, the theoretical R_{max} came up to be 251 RU assuming 1:1 interaction of EGF with sEGFR. This suggests that only 25% of the biosensor surface was either active or had immobilized ligands in accessible orientations.

For all sEGFR surface densities, the data from the primary analysis showed a poor fit (R>0.2) to 1:1 Langmuir interaction model suggesting that more complex binding mechanisms were operative. The curve fitting to the heterogeneous ligand- parallel intereactions model was significantly better indicating two independent binding sites in the system. For 3357RU of sEGFR immobilization, one of the two binding sites was a low affinity binding site which contributed to 90% of the total binding with an apparent k_a of $3.75e5M^{-1}s^{-1}$ and k_d of $0.0406s^{-1}$, resulting in a calculated K_D of 108nM which is in good agreement with the literature K_D for EGF and sEGFR interaction, as shown in Table 6.3-1. The high affinity binding site is just 10% of the total binding and has a calculated K_D of about 5.4nM. For 5500RU sEGFR surface density, the kinetic rate constants agreed

approximately to that of the previously obtained values except that the high affinity binding increased to 16% of the total binding.

Mass transfer limitations were tested with higher concentration of EGF (100nM) at flow rates 5µl/min, 15µl/min and 75µl/min. Figure 6.3-2 shows a representative Sensogram of EGF kinetics at three different flow rates. The kinetics of the binding of EGF to sEGFR changed less than a few percent with changes in flow rate, indicating that the reaction is not mass transfer limited.

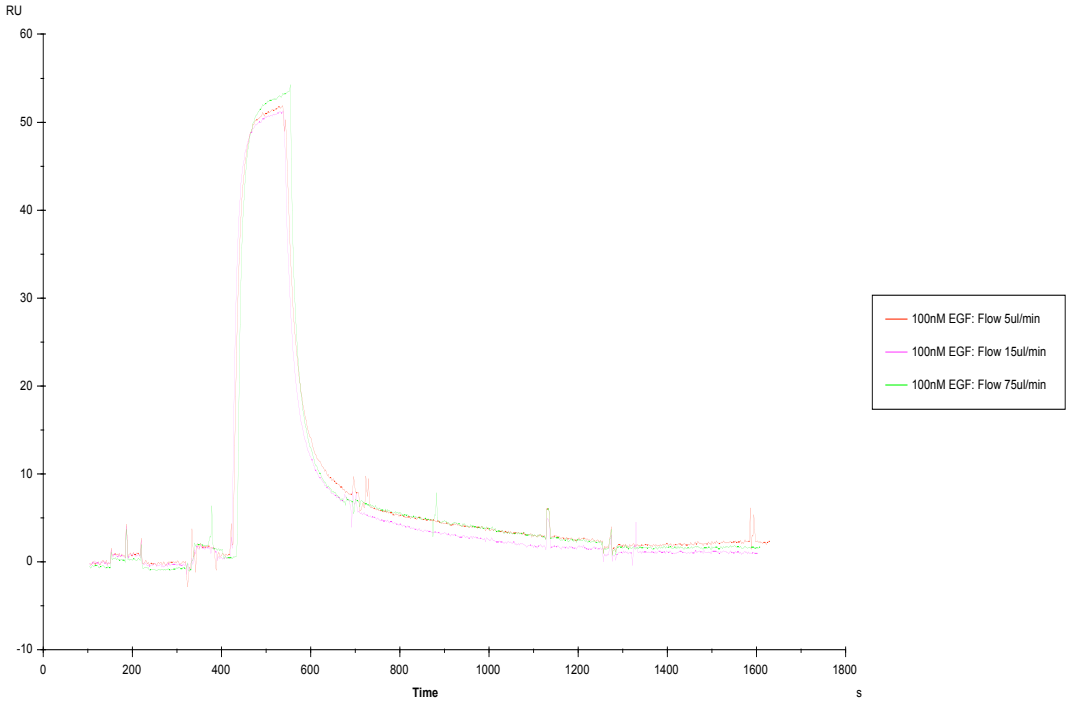

Figure 6.3-3 Data from Mass transfer control experiment. Kinetics of 100nM EGF at different flow rates (5µl/min, 15µl/min, and 75µl/min) was determined. Kinetics of EGF binding was same at all flow rates.

The equilibrium binding constants are reported in the Table 6.3-1 for EGF and sEGFR interaction. Comparisons are also given with that of literature values.

Table 6.3-1 Summary table for equilibrium binding constants for EGF/sEGFR interaction.

	Ligand	Analyte	Low Affinity Binding		High Affinity Binding	
			K_D (nM)	k_d (s^{-1})	K_D (nM)	k_d (s^{-1})
Our Studies (Biacore)	sEGFR	EGF	115±10	0.04	13±8	0.002
Iyer *et al*., 2007 (Biacore)	sEGFR	EGF	110	-	-	-
Shiqing Li *et al*., 2005 (Biacore)	EGF	sEGFR	130±3	-	-	-
Wade *et al*., 2002 (Biacore)	mEGF	sEGFR	400	0.042	28	0.02
Domagala *et al*., 2000	rhEGF	sEGFR	439	0.066	60	0.013
J Schlessinger *et al*., 1986 (In cells)	EGFR	EGF	1-10	-	0.01-0.1	-

6.3.2 Binding studies with Cetuximab

Cetuximab was used in increasing concentrations run from low order to high using kinetic analysis. HBS-EP buffer was used to make serial dilutions and the concentrations injected were 50, 20, 5, 2, 1, 0.2, 0.02nM. A 0nM sample was also injected for reference subtraction. The interaction was tested for mass transfer limitations using 0.2nM and 20nM concentrations at flow rates 5µl/min, 15µl/min and 75µl/min and the data obtained did not seem to be affected with change in flow rates indicating the reaction is not mass transport limited (data not shown). The data was analysed with varying concentrations of Cetuximab and two different surface densities of sEGFR (100 and 300RU). The representative Sensograms of evaluated data are presented in Figure 6.3-4.

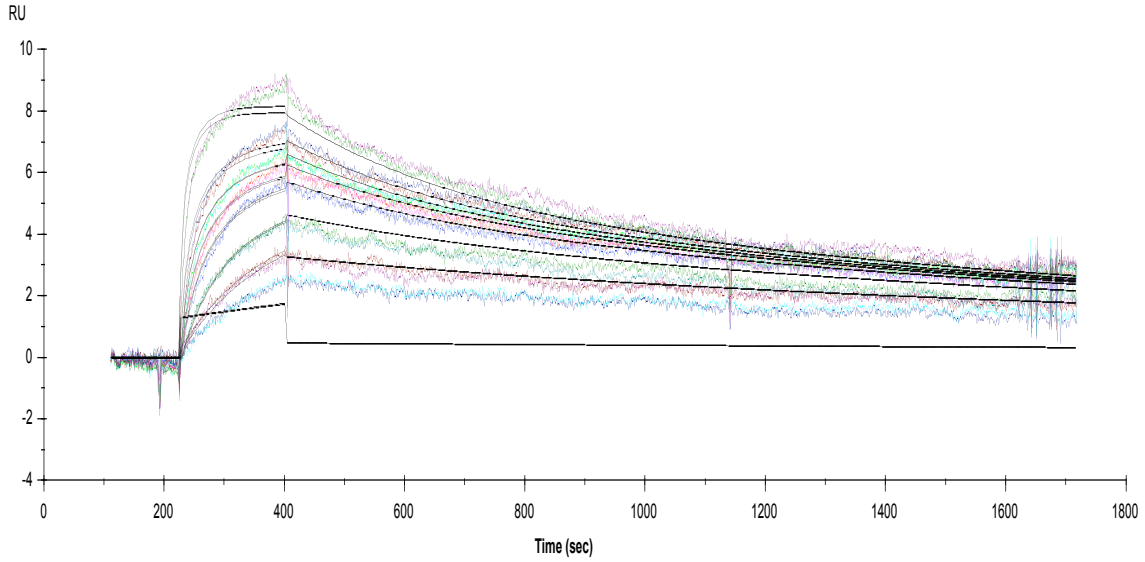

Figure 6.3-4 Kinetic analysis of C225 with sEGFR: various concentrations of C225 were injected at 30µl/min over 100 RU immobilized sEGFR and reference surface (activated and deactivated by EDC/NHS and ethanolamine). Curves shown are reference subtracted.

The Sensograms fitted using 1:1 Langmuir binding model showed significant deviations for higher surface density (data not shown). As the surface density was decreased to 100RU, 1:1 Langmuir binding model (data not shown) showed a better fit except that the higher and lower concentrations for analyte still showed deviations. However, the curve fitting was good using Bivalent analyte model (where the analyte can bind to two ligands simultaneously on the surface) for all surface densities. The curves showed a slow dissociation with apparent k_a of 8.36e5$M^{-1}s^{-1}$ and k_d of 1.81e-3s^{-1}, resulting in a calculated K_D of 0.2nM which is in good agreement with the literature.

The equilibrium binding constants for Cetuximab and sEGFR interactions are reported in the Table 6.3-2. Comparisons are also given with literature values.

Table 6.3-2 Summary table for equilibrium binding constants for Cetuximab/sEGFR interaction.

	K_D (nM)	k_d (s^{-1})
Our studies (Biacore)	0.15±0.05	0.002
Shiqing Li et.al, 2005 (Biacore)	2.3±0.5	-
Neil I. Goldstein et al., 1995 (Biacore)	0.2	-

6.3.3 Binding studies with P-13

P-13 was used in increasing concentrations run from low to high order using kinetic analysis wizard. HBS-EP buffer was used to serial dilutions and peptide concentrations of 1000, 800, 600, 400, 500, 300 and 200µM were injected to determine steady state binding affinity. The interactions were tested for mass transfer limitations using 400µM concentration at flow rates 5, 15 and 75µl/min and it did not seem to be affected by mass transport.

The Sensograms showed fast dissociation and the curves did not start to plateau to determine steady state binding affinity. The highest concentration (1000µM) seemed to show nonspecific binding and therefore was eliminated from analysis. The data was first fitted for dissociation and a k_d of 0.08±0.01s^{-1} was calculated. Considering the dissociation rate constant to be 0.08s^{-1}, data was fitted for an association constant k_a of 150$M^{-1}s^{-1}$ and a corresponding K_D of 500µM was observed for most curves. The fitted data for association as well as dissociation is shown in the Figure 6.3-5.

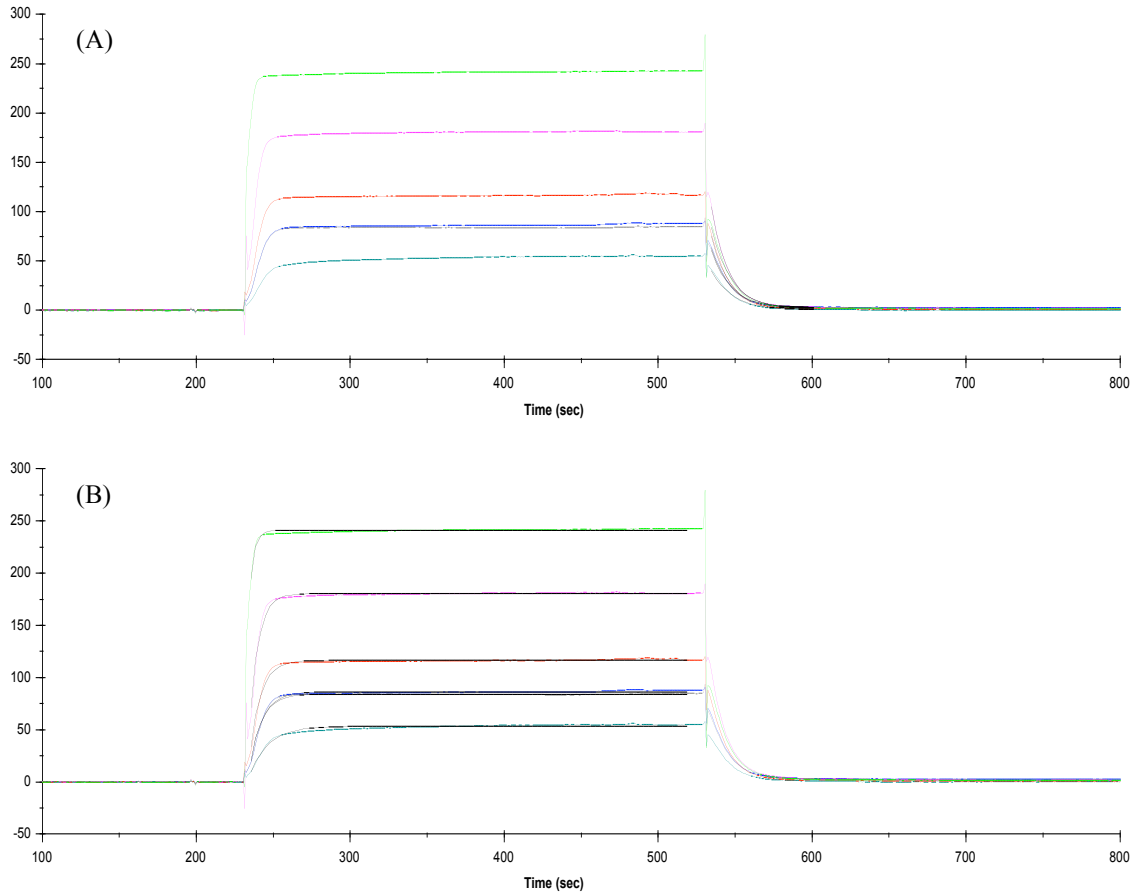

Figure 6.3-5 Kinetic analysis of P-13 with sEGFR. Data was fitted using various concentrations of P-13 (800, 600, 400, 300 and 200μM) injected at 30μl/min over sEGFR immobilized on the sensor surface. Kinetic rate constants were determined by fitting the data for dissociation (A) and association separately. Equilibrium dissociation constant was calculated from the obtained rate constants.

6.4 Discussion

We have determined that the dissociation rate constant for EGF binding to sEGFR is $0.04s^{-1}$ which is comparable to the rate constants shown in previous cell culture studies. Table 6.3-1 shows the equilibrium dissociation constant for EGF and sEGFR binding

assuming heterogeneous ligand parallel interactions model and is comparable with Iyer *et al*,. The affinity of sEGFR measured by Biacore is 100 fold lower than that in cells. This may be due to the fact that extra cellular domain of EGFR has much lower binding affinity than that of EGFR full length (Brown *et al.,* 1994). Also, deglycosylation of the recombinant protein used might have further decreased the affinity suggesting that the K_D values obtained using SPR studies may be 100 fold higher than measured in vitro.

There have been conflicting results on the binding stoichiometry of the EGF/EGFR interaction (Domagala *et al*., 1999). The binding stoichiometry using full length EGFR purified by affinity chromatography from A431 cells is reported to be 1:1; by Weber *et al*., 1984 in their studies. The later studies in cells, demonstrated that EGF binds to EGFR with two distinct dissociation constants: a minority (2%–5%) of high-affinity (K_D~0.1nM) receptors and a majority (95%–98%) of low-affinity (K_D~10nM) receptors (Schlessinger *et al*., 1986, 88). In Biacore studies carried over by Domagala *et al*., they reported that global analysis of EGF/sEGFR binding curves indicated that a simple 1:1 interaction did not adequately describe the experimental data, and that more complex interactions were operative. Additionally, competition analysis of the data obtained using either direct measurement of free sEGFR using Biacore or Fluorescence Anisotropy experiments yielded biphasic Scatchard plots indicative of multiple binding sites, with 10-15% of the high affinity sites (Domagala *et al*., 2000).

In our studies, we immobilized sEGFR in order to closely imitate the natural system. The flexibility of the dextran matrix and high local concentrations of immobilized

receptors made it possible to form complexes that mimic the cell surface better than solution phase assays (Myszka *et al*., 1999). Nonetheless, the data fitting using simultaneous k_a/k_d model indicated a two state binding of EGF and sEGFR with a 90% low affinity site with K_D of 115±10nM which is consistent with K_D of EGF binding to sEGFR established in Li *et al.,* and Iyer *et al*. The other 10% is high affinity binding and the physical reasons causing this are unclear.

Since Cetuximab is a bivalent monoclonal antibody, data fitting for C225 interaction with sEGFR was done using bivalent analyte model. The dissociation constant was shown in Table 6.3-2 in comparison with literature. We immobilized sEGFR on the surface and made C225 to flow on the ligand. When the surface density of sEGFR was 100RU, the data fitted well to 1:1 Langmuir model. As surface density of sEGFR was increased, fitting showed significant deviations. This may be due to the increased avidity effects on the surface. Avidity could be described as follows. If an antibody is in solution, it has the potential to cross-link with two antigens on the surface. This will result in an apparent higher affinity and the kinetics cannot be described with a simple interaction model (Myszka *et al*., 1999). Studies by Shiqing Li *et al*., showed that Cetuximab binds to sEGFR with a dissociation constant of 2.3nM which is 10 fold greater than what we got in our studies. This may be due to the difference in experimental methods adopted and the nature of the Cetuximab used. Li *et al*., used a monovalent Fab fragment in their study where as we used bivalent antibody. Also, in our studies, we reported the dissociation rate constant for immobilized sEGFR with Cetuximab, as an analyte, regeneration was possible in our system. This was practically not possible with sEGFR binding to immobilized Fab

as the sEGFR sticks very tightly to the Fab and never comes off under normal buffer conditions (Li *et al*., 2005).

P-13 interactions with sEGFR showed a binding with significantly lower affinity as compared to the prototypical ligands such as EGF. The K_D of the peptide is reported to be 500μM and the kinetic dissociation rate constant is $0.08\pm0.01s^{-1}$. Although the equilibrium dissociation constant is way too high for comparison, the dissociation rate constant seems to be comparable to a nano molar binder, Hydroxybenzylpindolol (Rimon *et al*., 1980). As the association rate constant is very low, the peptide is rendered as a very weak binder. This suggests that the binding region of the peptide on the receptor could be highly inaccessible. We may conclude from this study that target location plays an important role for peptide therapeutics. In our study, to get a more promising binder, the upper regions of domain III must be considered as target.

CHAPTER 7 Future Work

- In order to obtain more reliable kinetic rate constants and equilibrium binding constant for the P-13-sEGFR interaction, it is necessary to repeat the Biacore experiments more number of times with higher concentrations of peptide. Due to the limited availability of resources, we had to optimize our experiments.

- To obtain a wider knowledge on interaction of the peptide P-13 with the receptor and to determine if the peptide has any inhibiting effect on EGFR cell signaling, it is recommended to carry on cell studies with the peptide.

- In order to explore the structure of high affinity binding of EGF and sEGFR, it is suggested to recover the EGF-sEGFR complex and carry forward x-ray crystallography studies on the complex.

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